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Cartilage repair using new polysaccharidic biomaterials: macroscopic, histological and biochemical approaches in a rat model of cartilage defect

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Summary

Objective: The present study aims at evaluating, in a rat model of cartilage defect, the potential of various polymers as filling and repair biomaterials. The macroscopic and histological observations are compared to biochemical parameters in order to appreciate the pertinence of the latter as suitable criteria in tissue engineering.

Methods: A hydrogel, consisting of hyaluronic acid (HA), covalently substituted by hydrophobic alkyl chains (HA12, HA18) and an alginate sponge, alone (Asp) or combined with HA (AHAsp) or combined with HA and chondrocytes (HYBsp) were evaluated. Cartilage lesions were drilled in femoral trochlea of rats. The analyses were performed on trochlea as well as on patella and condyles.

Results: Repairs achieved with hydrogels had a similar macroscopic appearance than those afforded by AHAsp and HYBsp. Best macroscopic and histological scores were obtained with HA18 and HYBsp in comparison with alginate group ($P<0.01$ and $P<0.02$ respectively). Biochemical evaluations confirmed the presence of similar amounts of proteoglycans in the repaired zones and in the controls, though with different ΔdiC4S/ΔdiC6S ratios and enhanced HA levels.

Conclusions: Hydrogels or sponges proved to be colonized by cells synthesizing a matrix with a high HA content. The matrix obtained eventually turns hyaline and takes over the scaffold. The addition of HA and/or chondrocytes to Asp significantly improves the macroscopic and histological scores ($P<0.05$ and $P<0.02$ respectively). However, biochemical parameters are significantly different of those evaluated in native cartilage. The present study shows that only biochemical parameters allow to discriminate between various biomaterials in tissue engineering and are essential informations which should be taken into account in addition to macroscopic and histological observations.

Key words: Cartilage repair, Hyaluronic acid, Alginate, Chondrocytes.

Abbreviations: AHAsp, Alginate plus hyaluronate sponge; Alg, Sodium alginate; Asp, Alginate sponge; bFGF, basic fibroblast growth factor; BMP-2, Bone morphogenetic protein-2; CG, Control group of rats; ΔdiC4S, Unsaturated disaccharide of 4-sulfated chondroitin; ΔdiC6S, Unsaturated disaccharide of 6-sulfated chondroitin; ΔdiHA, Unsaturated disaccharide of hyaluronate; DMB, dimethylmethylene blue; GAGs, sulfated glycosaminoglycans; HA, Sodium hyaluronate or hyaluronic acid; HA12, HA backbone grafted with alkyl chains of twelve carbons; HA18, HA backbone grafted with alkyl chains of eighteen carbons; HES, Hematoxylin eosin safran; HYBsp, Hybrid sponge containing alginate, hyaluronate and autologous chondrocytes; IGF-1, Insulin-like growth factor-1; SR, Spontaneous repair group of rats; TGF-beta-1, Transforming growth factor-beta-1.

Introduction

The repair of cartilage is a topic of current actuality for scientists, surgeons and patients, as well as an important concern in terms of economic and human costs. Several centuries after its first observation, this problem of cartilage repair has not yet found a satisfactory and definitive answer. The self-repair potential of this tissue is weak since cartilage is not vascularized and chondrocytes divide slowly. Full-thickness defects are mostly post-traumatic. Their natural evolution to mechanically unsatisfactory fibrocartilage illustrates the insufficiency of chondrogenesis[1]. However, the recruitment of new cells so as to improve the neo-synthesized cartilage matrix is possible[2]. Several sites can provide cells for the induction of cartilage repair: chondroblasts, perichondrial cells or mesenchymal stem cells[3,4]. Hunziker *et al.*[5] also proposed the recruitment of cells issued from the synovial membrane.

Different methods are currently used for the treatment of acute cartilage lesions[6,7], though with limited success in so far as they do not prevent the long-term evolution to osteoarthritis (OA)[3,8]. Symptomatic and non-specific treatments are nevertheless always useful. For example, continuous passive motion with initial non-weight-bearing often generates a clinical improvement which, unfortunately, may turn out to be not sustainable with time. Similar problems

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are met with marrow-stimulation techniques, generally affording an insufficient clinical outcome[9]. Other more sophisticated techniques (mosaicplasty, periostal or perichondrial autografts . . .)[8,10] consist in tissue or cell grafting. However, both the limited availability of tissue and the difficulties of bonding hamper the usability of cartilage autografts.

Some other methods, proposed for partial or full-thickness defects, aim at seeding the defect with cells capable of synthesizing a new cartilage. This latter should be hyaline, well integrated to native cartilage and exhibit similar mechanical properties. In the method described by Brittberg *et al.*[11,12], cell suspensions are transplanted in the defect and restrained by a sutured periostal flap. However, the presence of mixed collagen types (I and II) was sometimes detected by histology and sutures were suspected to cause focal degenerative loss of proteoglycans[13]. This procedure has been recently modified so as to circumvent these drawbacks[14]. Yet, the long-term clinical outcome is unknown.

Hybrid biomaterials are in theory always more attractive[15–19]: they surround chondrocytes and offer protection against immunity rejection and phenotype instability. They can also present some advantages for the survival, proliferation, differentiation and synthesis ability of chondrocytes, thus able to synthesize matrix components and to reconstitute the hyaline architecture of cartilage[20]. Chondrocytes are issued from cartilage by enzymatic digestion[21–23] or from medullar stem cells by differentiation[24,25]. In most reports, chondrocytes are cultivated in monolayers for 15–21 days prior to their seeding into a considered biomaterial. In these conditions, they can proliferate, but they also dedifferentiate into cells presenting a fibroblastic-like phenotype, accompanied by a decreased synthesis of type II collagen and proteoglycans[26]. Fibroblast-like cells can redifferentiate when placed in a three-dimensional system[27]. This latter must be chosen for its biological and structural properties. It must also be biocompatible, sterile, porous (to accommodate cell proliferation) and, preferably, bioresorbable. Numerous non-degradable and degradable polymers, recently reviewed by Seal *et al.*[28], have been studied. For example, some biomaterials are naturally-derived materials like thrombin[29,30], collagen[16,17,20,26,31], hyaluronic acid (HA)[15,18] and its chemical derivatives[32–34], agarose[35,36], alginate[25,37–41], or chitosan[42]. Some others are synthetic degradable polymers, such as polyesters (e.g. polylactic acid, polyglycolic acid)[21,23,43,44], polyethylene oxide[45] or injectable polymers like poly(ethylene glycol)[46,47].

The addition of growth factors (e.g. IGF-1, bFGF, TGF-beta-1 or BMP-2)[48–50] in implanted materials constitutes a new field of investigation aiming at an enhanced efficacy. Recently, Caterson *et al.*[51] observed a best chondrogenic differentiation of bone marrow-derived cells seeded in a poly-L-lactic acid/alginate three-dimensional scaffold treated with TGF-beta-1. The ability of growth factors to differentiate stem cells into chondrocytes and to form cartilage has been clearly shown, but efficient and harmful doses still have to be specified before that human applications can be envisaged.

In the present work, several biomaterials have been investigated for cartilage substitution in an animal model. The goal was to repair full-thickness defects of rat articular cartilage. Similar lesions in man are known for their spontaneous evolution to OA. The assessment of various filling materials (hybrid sponge, gels and sponges without cells) was performed according to clinical, histological and biochemical criteria.

Materials and methods

BIOMATERIAL PREPARATION

Different types of biomaterials, gels or sponges, were tested. They were prepared from sodium alginate (Pronova, Norway, *Laminaria hyperborea*, ultra-pure, guluronate/mannuronate ratio=70/30) and from hyaluronic acid issued from bacterial fermentation (Acros, U.S.A., 450 000 Da, water 15%). Hydrogels used in the present study consist in hydrophobically-associating derivatives of HA, prepared by covalent immobilization of long alkyl chains (in HA12≈10 moles C12 chains/100 moles glucuronic acid unit and in HA18≈3.6 moles C18 chains/100 moles glucuronic acid unit) onto the polysaccharide hydrophilic bakbone. In semi-dilute solution (C≈5 to 10 g/L), hydrophobic alkyl chains associate intermolecularly, leading to the formation of three-dimensional physical networks, exhibiting the rheological properties of high viscosity viscoelastic-to-elastic hydrogels [$\eta_0 \approx 5\times10^3$ to 5×10^5 fold that of native HA at the same concentration (10 g/L in 0.15 M NaCl)][52,53–55].

Sponges were prepared by freeze-drying of an aqueous mixture containing alginate (2% w/v) combined or not with HA (0.5% w/v) (Asp and AHAsp), as described by Shapiro *et al.*[56] The physicochemical properties of these porous scaffolds have been previously studied by Miralles *et al.*[40]. The hybrid biomaterial, based on AHAsp, and seeded with autologous chondrocytes will be named Hybrid sponge (HYBsp). Chondrocytes were harvested under general anesthesia from cartilage of xiphoid appendix. They were taken and hydrolyzed by pronase E (2 mg/g tissue), 2 h, 37°C (*Streptomyces griseum*, Sigma, U.S.A.), then by collagenase B (1.5 mg/g cartilage), 8 h, 37°C (*Clostridium hystolyticum*, Boehringer, Germany). After digestion, chondrocytes were sufficiently numerous to be directly included in sponges at a level of 5×10^4 cells/sponge. The sponges were then cultivated for 15 days before implantation in Nut-Mix F12 medium [500 ml supplemented with fetal calf serum (55 ml), gentamycine (4 ml), amphotericin B (0.5 ml) and L-glutamine (6 ml)]. All chemicals were purchased from Gibco, France.

The sterilization of all biomaterials was carried out by wet heating (20 min at 121°C).

ANIMAL EXPERIMENTATION

A medial parapatellar arthrotomy was performed under general anesthesia in the left knee of 5–6-week-old rats. A full-thickness cartilage defect of 1.3 mm diameter was drilled in the femoral trochlea. Filling of the resulting defect was achieved by injection of a hydrogel or insertion of a piece of sponge. After this implantation, the patella was put back in its place, thus permitting the lesion to be covered and the biomaterial within to be maintained. The right knee constituted an individual control of surgery, but without cartilage lesion. In the same time, all the animals were intraperitoneally equipped with a transmitter in order to monitor their spontaneous locomotor activity and their body temperature during 10 days following the implantation of biomaterials. The experimental procedure of this biotelemetric study was previously described by Gegout-Pottie *et al.*[57].

Table I

Macroscopic and histological evaluation of tissue repair, 10, 20 and 40 days after implantation of biomaterial (HA12 and HA18: HA derivatives obtain by grafting of alkyl chains C12 or C18; Asp: alginate sponge; AHAsp: alginate sponge combined with HA; HYBsp: alginate sponge combined with HA and autologous chondrocytes) or spontaneous repair (SR)

Group	Day	*N*	Macroscopic score/12 (1)	*N*	Histological score/20 (2)	Statistics (3)
SR	10	3	3.3±0.9	4	4.5±1.2	
	20	3	2.0±1.0	4	9.0±3.0	NS
	40	4	9.2±0.7	2	10.5±1.5	
HA12	10	3	4.3±2.4	2	9.5±1.5	
	20	4	3.0±0.7	4	7.5±1.4	NS
	40	3	10.3±1.2	4	7.7±1.8	
HA18	10	4	5.2±1.0	2	8.0±1.0	
	20	4	4.0±0.9	2	9.5±1.5	***
	40	4	10.7±1.0	4	11.5±1.2	
Asp	10	3	3.0±2.1	4	5.5±2.9	
	20	4	2.2±0.8	4	9.7±1.4	
	40	3	6.0±2.1	2	8.5±2.5	
AHAsp	10	3	1.7±1.2	4	4.7±1.5	
	20	4	3.0±0.7	4	11.5±1.3	*
	40	3	10.7±0.9	4	10.2±1.3	
HYBsp	10	4	6.7±1.8	4	4.7±1.5	
	20	4	6.5±1.7	2	8.0±1.0	**
	40	4	9.7±1.1	2	14.0±1.0	

(1) Global macroscopic score/12: means±S.E.M. issued from *N* animals observed for five parameters (surface regularity/2, resurfacing/3, junction aspect/2, repair aspect/2 and fill up/3).

(2) Global histological score/20: means±S.E.M. issued from *N* observations realized by two manipulators for eight parameters (cell morphology/4, subchondral bone/3, filling/2, surface regularity/1, junction/2, proteoglycan staining/4, collagen fibers/4).

(3) Data were analysed in a two-way ANOVA (groups vs macroscopic and histological scores) and significance of differences (Asp vs other groups) is set at $P<0.05$; ND: non-determined; NS: non-significantly different; $P<0.05$*; $P<0.02$**; $P<0.01$***.

One hundred and eight animals with a defect and one control group of *N*=13 animals without defect were used for the study. Joint defects were treated according to different procedures: in the spontaneous repair group (SR) no scaffold was introduced (*N*=18). In HA-gel groups, lesions were filled with HA12 gel (*N*=18) or HA18 gel (*N*=18). In sponge groups, lesions were repaired by a piece of Alg sponge (Asp) (*N*=18), Alg combined with HA sponge (AHAsp) (*N*=18) or Hybrid sponge (HYBsp) (*N*=18). During the experiment, the body weight of implanted and control animals was monitored at various intervals.

Sacrifice occurred at days 10, 20 and 40, and both knees were collected. In every group of six rats, four were used for biochemical analysis and two for histological assessment. Synovial fluids were collected by absorption on filter papers. The repaired areas were macroscopically assessed according to a semi-quantitative scale, as described in Table I.

HISTOLOGICAL EVALUATION

Histological routine methods, involving 3 days fixation in formalin (10% v/v in formaldehyde, pH 7.4) (Sigma, France), followed by 3 weeks decalcification in ethylene diamine tetracetic acid (Merck, Germany) (10% v/v in formalin), were used. After dehydratation, joints were embedded in paraffin and cut on a rotary microtome at 5 μm sections. These sections were stained with Hematoxylin Eosin Safran (HES) for global anatomic analysis, with Toluidin blue for proteoglycans staining and with Sirius red for collagen network architecture. Slides were graded by two operators in the same conditions. A semi quantitative scale, adapted from Caplan *et al.*[2], with new assessment criteria concerning remnants of implanted matrix (HES staining) and collagen architecture (Sirius red staining examined under polarized light), was used. With this latter staining, normal hyaline cartilage was structured in three layers: a thin superficial red, an intermediate black and a thick deep green, accompanied by Gothic arcs surrounding dark spaces for chondrocytes. Moreover, the Toluidin blue staining of the matrix was evaluated as a percentage of the matrix staining measured on control animals of the same age, by digital computer (N.I.H. picture v1.62, W. Rasbaud, National Institute of Health, U.S.A.). The whole staining and digital session were performed on the same day by a single operator. Addition of all items, except remnants of matrix (irrelevant for the SR group), led to a total score on 20, as described in Table I.

BIOCHEMICAL EVALUATION

In order to evaluate the quality of repaired cartilage, fast turnover components, HA and sulfated glycosaminoglycans (GAGs), were measured. After macroscopic assessment, different zones of femoral cartilage (repaired zone and weight-bearing cartilage) were collected with a 2 mm Ø biopsy punch. Each patella was decalcified in 1.5 ml of 5% formic acid (Merck, Germany) for one night before its cartilage was peeled. The samples were dried overnight at 37°C (one punch for the repaired zone, six punches for weight-bearing cartilage, and 0.5 to 1.5 mg of cartilage for patella). They were digested at 60°C during 24 h by 10 μl of

aqueous solution of papain (6 mg/ml) (Sigma, France) into 200 μl of buffer (pH 6) [20 mM anhydrous Na_2HPO_4 (Merck, Germany), 1 mM EDTA (Merck, Germany) and 2 mM dithiothreitol (Sigma, France), completed to 100 ml with distilled water]. Hydrolysis was stopped by addition of 10 μl of 220 mM sodium monoiodoacetate (Merck, Germany). Then the volume was adjusted to 400 μl with 50 mM Tris-HCl (pH 8) buffer (Merck, Germany).

GAGs analysis

The assay is based on the ability of GAG's sulfate groups to bind to the cationic dye dimethylmethylene blue (DMB) at pH 3[58]. At the concentrations used, there was no interaction with alginate, when present in solution[59]. 100 μl of papain-hydrolyzed samples were added to 2.5 ml of DMB [0.016 g/l of DMB (Aldrich, France), 3.04 g/l of glycine (Sigma, France), 2.37 g/l of NaCl (Sigma, France), 95 ml/l of 0.1 M HCl (Merck, Germany)]. Quantification of GAGs was performed using a reference curve, constructed in the same conditions with chondroitin 4S (0.25 to 10 μg/ml) (Sigma, France).

DNA analysis

The fluorimetric assay used was previously described by Lipman[60]. Briefly, 25 μl of papain-hydrolyzed solution were added to 25 μl of buffer (pH 7.4) (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl) and 2 ml Hoechst Dye 33258 (0.1 mg/ml) (Hoechst, Germany). Readings, performed at λ_{exc}=356 nm and λ_{em}=458 nm, were compared to a reference curve constructed with calf thymus DNA (0.01 to 2 μg/ml) (Sigma, France).

Unsaturated disaccharides analysis in cartilage

Unsaturated disaccharides of chondroitin (ΔdiC4S, ΔdiC6S) and hyaluronic acid (ΔdiHA) were analysed by capillary zone electrophoresis as described by Payan *et al.*[61]. Briefly, 25 μl of chondroitinase ABC (1 UI/l) (Sigma, France) and 150 μl of Tris buffer (pH 8) were added to 225 μl of papain-hydrolyzed samples. The mixtures were maintained at 37°C overnight. 250 μl aliquots were then filtered on Ultrafree-MC (Millipore, U.S.A.) at 10 000 g for 1 h. 150 μl of the filtrates, diluted or not in Tris buffer, were completed with 10 μl of internal standard solution. Unsaturated disaccharide levels were estimated vs a calibration curve established with a standard mixture of ΔdiC4S and ΔdiC6S purchased from Sigma (France) and ΔdiHA obtained after enzymatic hydrolysis of rooster comb Hyaluronate (Sigma, France).

Determination of HA in synovial fluids

This analysis was performed using a high-performance liquid chromatography method described by Payan *et al.*[62]. Filter papers with absorbed synovial fluids were placed in 100 μl of 0.9% NaCl and 10 μl of *Streptomyces hyaluronidase* 100 UI/l (Sigma). Then, 100 μl of 20 mM acetate buffer (pH 6) were added. After 48 h of hydrolysis at 20°C, 10 μl of internal standard solution were added. HA standards at different concentrations were prepared similarly to establish the calibration curve.

STATISTICAL ANALYSIS

Data were expressed as mean±standard error of the mean (S.E.M.).

Residual percentage of HA in left or right knee of treated groups are obtained by the following relation [1]:

$$\frac{(\text{HA level in treated group} - \text{HA level in CG})}{\text{HA level in CG}} \times 100$$

One or two-way ANOVA was used to determine the statistical significance of the differences observed at various times between left and right knees of the different groups. *P* values smaller than 0.05 were considered significant.

Results

BEHAVIOURAL STUDY OF THE ANIMALS

The follow-up of animals by biotelemetry was performed during 10 days after the implantation. No major variations of the mobility and the body temperature of animals (36–38°C) were evidenced during this period.

A slight decrease of the rat activity, likely due to the general anesthesia conditions used, was observed during the first 48 h after the surgical intervention. All animals proved to move without handicap during their displacements, owing to the localization of implanted lesions in a non-load-bearing area (femoral trochlea).

Animals were weighed at regular intervals throughout the experiment. The average weight of the implanted rats was identical to that of the same age control group, at all measuring times from day 0 to day 40. The rats were therefore capable of standing up on their implanted legs in order to eat and to drink.

MACROSCOPIC AND HISTOLOGICAL ANALYSES OF THE REPAIR TISSUE

The visualization of repaired areas shows an optimal filling of the defect since D20 for HA18, and HYBsp (Fig. 1). In contrast, this filling is never complete for other biomaterials. The global macroscopic score obtained for gel-repaired defects was similar to that of sponge-repaired ones (Table I). These results were confirmed by the global histological score (Table I) assessing anatomical performances of biomaterials. For all groups, this score improved with time. As for macroscopic assessment, HA18 had the best histological score at D40 among gels (HA18 11.50/20 and HA12 7.75/20). Among sponges, HYBsp had the best score at D40 (14/20 compared to Asp 8.50/20 and AHAsp 10.25/20) (Fig. 2).

The histological study has shown that at Day 40, hyaline-like areas were present only in HA18 and HYBsp groups [Fig. 3(a), (c) respectively]. Moreover, proteoglycans staining was superior in the HYBsp group.

The sponges were slowly degraded and remained visible after D40, as small fragments surrounded by collagen fibers [Fig. 3(d)]. Chondrocyte-like cells were visible inside those fibers.

For HA18 or HYBsp, repaired tissue tends to turn hyaline with time, with increasingly numerous cells and organized collagen fibers [Fig. 3(b), (d) respectively].

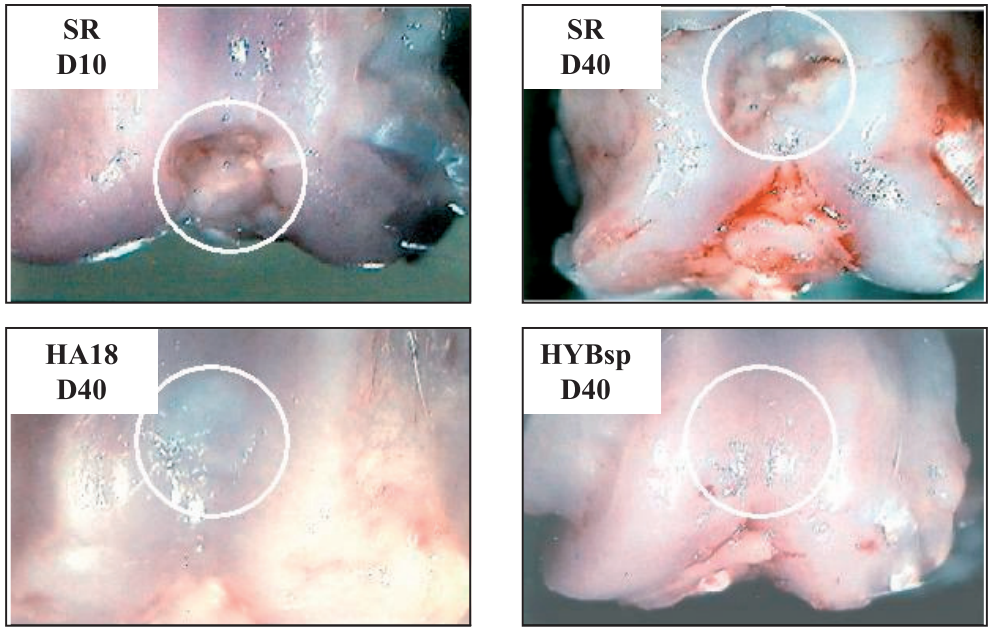

Fig. 1. Macroscopic analysis of repaired areas (×10). Bad surface reconstruction with the SR group at D10 and D40. Good resurfacing with HA18-gel at D40. However, at the view of respective stainings, the repaired zone is still distinguishable from native cartilage. Optimal resurfacing for HYBsp group at D40.

CELL REHABITATION

DNA measurements in repaired zones confirmed the presence of cells in the different materials. Since D10, DNA levels recovered in the trochlea of surgically-treated knees, whatever left repaired or right control, are similar to those observed in trochlea of absolute controls (CG) (2.0±0.3 μg/punch; *N*=25).

HA ANALYSIS

This analysis was performed on the synovial fluid and on a calibrated punch of trochlea, taken from both left and right knees of animals, implanted or not (self-repair) and from control animals left without any surgical intervention. At Day 10, 20 and 40, whatever the sample, average HA amounts in the synovial fluid of absolute controls (CG) (3.3±0.4 μg/joint for *N*=20) were not significantly different of those taken from control right knees of operated animals (Fig. 4) [varying from 1.8±0.3 μg/joint (*N*=4) to 4.6±0.6 μg/joint (*N*=4)].

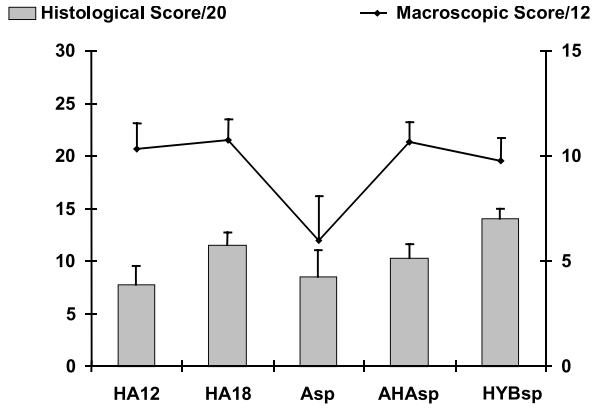

Fig. 2. Comparison of macroscopic score/12 (right axis) and histological score/20 (left axis). Data are presented as a mean±S.E.M. for *N* observations and five parameters (macroscopic score) or *N* observations and eight parameters (histological score), respectively, performed by two manipulators (see Table I).

In control right trochlea of treated rats, the percentage of residual HA, calculated with the relation [1], varied by a factor 0.5 to 1, as compared to the average value estimated for animals of the CG group (1.6±0.1 μg/punch) (Fig. 4). For these synovial fluid and trochlea control samples, HA ratios remained relatively stable with time since no significant difference was evidenced between days 10, 20 and 40.

Significant differences in synovial HA were observed, depending on the nature of the treatment (self repair or scaffold implantation). Alginate-based sponges and SR samples single out more particularly since average synovial HA may reach values from 5.0±1 μg/left joint vs 2.7±1 μg/right joint up to 10.3±2.0 μg/left joint vs 4.6±1.2 μg/right joint, at D40 on the implanted left side. This HA hyperproduction suggests that, in these repair conditions, the metabolism is disturbed. This phenomenon is not observed with HA12 and HA18 samples, indicating that there is no massive release of these polymers in the joint cavity, triggering undesirable local effects.

HA measurements in repaired trochlea clearly evidenced an hyperproduction of this polysaccharide at day 10, reaching values more than three-fold higher than those of absolute controls (CG) (Fig. 4). Such HA amounts in repaired areas can only result from a colonization of the biomaterial by neighbouring cells capable of synthesizing HA. For all samples, HA amounts decreased with time to reach normal values at day 40.

GLYCOSAMINOGLYCANS ANALYSIS

Amounts of total sulfated glycosaminoglycans (GAGs) as well as chondroitin sulfates 4S to 6S ratios were

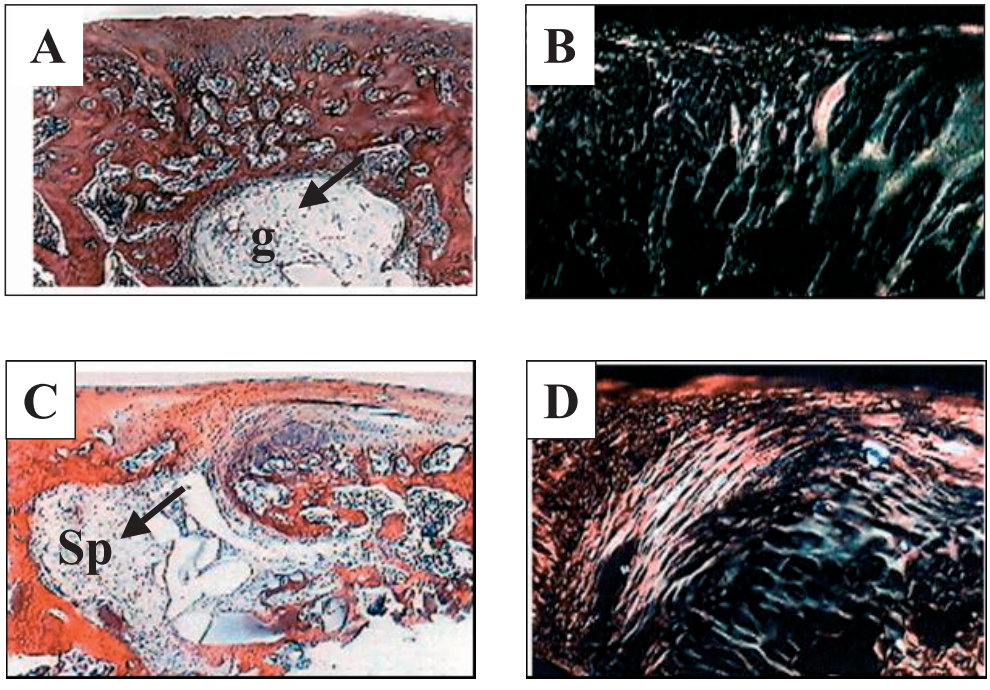

Fig. 3. Histological analysis of repaired areas (×40) at D40, for HA18 gel implant (a) and (b) and HYBsp (c) and (d). (a) HES staining: visible gel colonized by some cells (lower layer); a few zones of hyaline tissue (upper layer); remnants of gel surrounded by fibrocartilage (intermediate layer). (b) Sirius red staining examined under polarized light confirms the presence of organized collagen fibers. (c) HES staining: visible sponge with numerous cells (lower layer); fibrocartilage zones are still distinguishable in the intermediate layer. (d) Sirius red staining examined under polarized light: some collagen fibers are visualized in the vicinity of the native cartilage.

evaluated in the various zones (controls or repaired) of joint cartilage (trochlea, condyle and patella).

For all groups, data obtained for control right knees of treated animals were systematically compared to those of the CG group, in order to take into account the potential individual variations, and also to appreciate a possible long-range effect of biomaterials and/or surgery on other cartilage sites surrounding the implant.

GAGs levels observed in repaired trochlea at D10 are significantly different of those determined in right knees in the whole group of treated animals (Fig. 5). Whereas in the SR, HA12 and HA18 groups an incomplete matrix synthesis is observed, AHAsp and HYBsp implants lead to a matrix hyperproduction (Fig. 5). This phenomenon suggests a superiority of HA-based sponges, as compared to the HA-gels, that can be attributed to their porous structure. For the HYBsp group, the fifteen days of *in vitro* matrix preparation before its implantation leads to an increase of the GAGs levels in the repaired zones of this group. Owing to the large individual variability of this parameter in control knees as well as in treated ones, no clear-cut conclusion can be discerned. Such variability makes this parameter inappropriate as only criteria to appreciate the quality of cartilage repair with biomaterials, whatever their nature.

The measurement of chondroitin sulfates, especially their 4S to 6S ratios appears as a more discriminative parameter. This indicator is significantly different for all samples of operated animals, for the repaired trochlea and for the patella facing them (Fig. 6). Whatever the repair mode, self-repair or scaffold, hydrogel or sponge, hybrid material, presence or absence of HA, this indicator, which varies with the age of animals, never reached the values obtained neither with the absolute control (CG) (D10: 22.0±2.4; D20: 18.0±1.2; D40: 12.6±0.8) or with the control knees of opposite legs (Fig. 6).

The hybrid implant of the HYBsp group systematically disturbed the ΔdiC4S/ΔdiC6S ratio of both left and right sides, suggesting a paracrine effect of this type of implant.

In repaired trochlea, the decrease of the ΔdiC4S/ΔdiC6S ratio was due to the variation of the chondroitin 4S, systematically inferior in the treated knee. In contrast, in the case of patella, this ratio was different owing to a significant increase of the chondroitin 6S, as compared to that in control knees.

Discussion

The present work focused on the *in vivo* study of cartilage repair in the presence of various polysaccharidic scaffolding biomaterials. The diversity of repair processes proposed in the literature and of tools of assessment is a major drawback which led us to envisage the study on our various biomaterials within a single experimental rat model and using the same investigation tools. The capacity of hydrogels or sponge materials to repair a focal lesion of cartilage was evaluated. Also, the potential interest of the incorporation of hyaluronate or autologous chondrocytes was investigated.

All filling materials, except one (Asp), were prepared with HA or its hydrophobic derivatives. HA is a non-sulfated polysaccharide of the natural cartilage matrix. It forms aggregates of proteoglycans *via* a link-protein. It can also bind chondrocytes through receptors (CD44 for example), thus improving cellular adherence and cell-matrix interactions[1,15,18,32]. Hydrophobic derivatives used in this study were obtained by chemical modification of native HA, in order to enhance its rheological properties[52,53].

Sponges were prepared with alginate (Alg), alone or combined to HA. Alg is a polysaccharide widely used for its

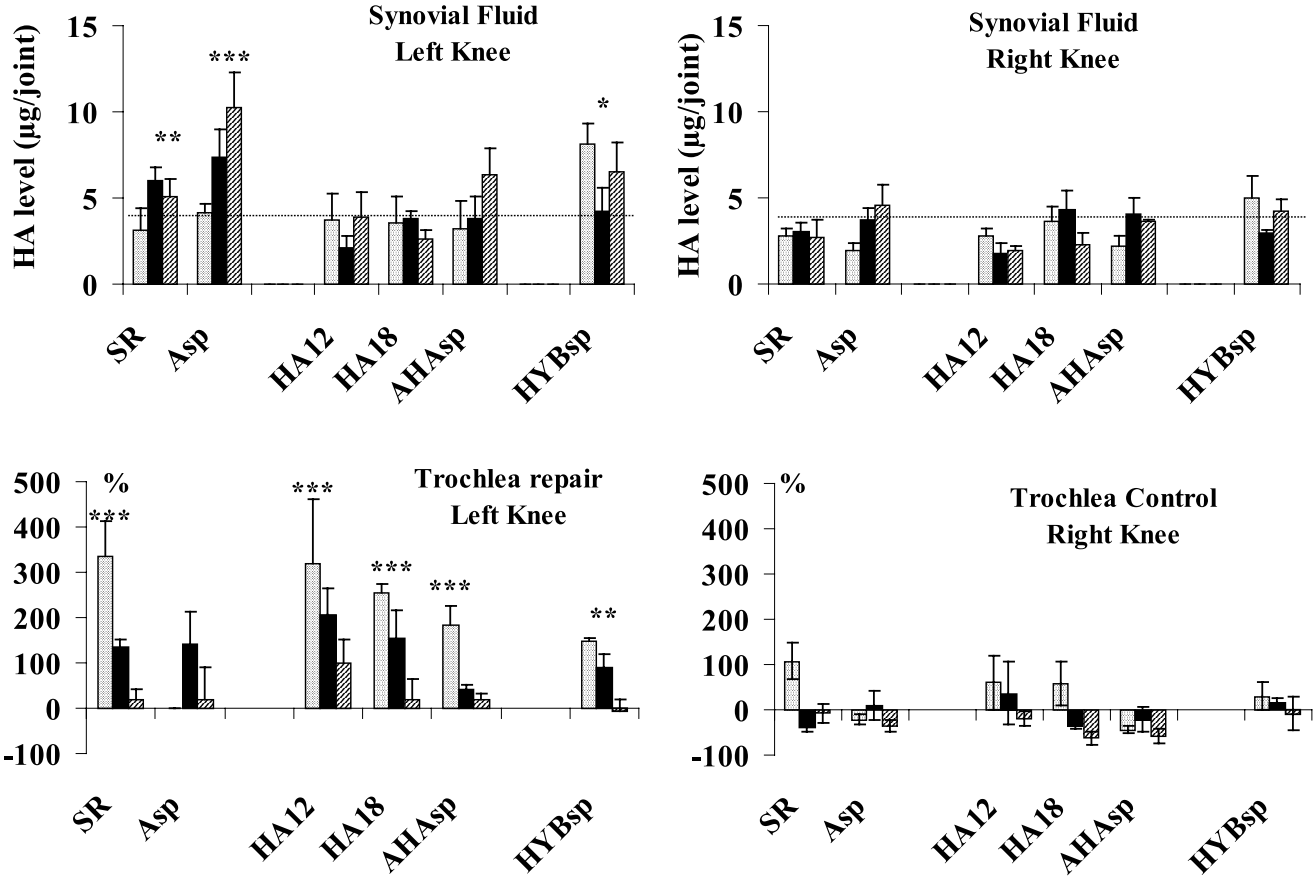

Fig. 4. HA measurements in synovial fluid and trochlea of both knees at D10 (▨), D20 (■) and D40 (▧). Analysis of HA is described in the Materials and methods section. The mean value of synovial fluid HA is expressed in µg of HA/joint and is obtained from left and right knees of the CG group (· · ·). Residual percentages of HA in trochlea are calculated from relation [1] (see Materials and methods). Data are expressed as a mean value±S.E.M. for N=4. Statistic analysis is performed by two-way ANOVA to test the significant difference between right and left knees of both groups with P<0.05*; P<0.02**; P<0.01***.

excellent structural properties: in the presence of divalent cations (Ca^{2+}), it forms a cohesive and firm gel, the properties of which vary with the viscosity, purity and source of the powder, as well as with the method of gel preparation. The optimal proportion of HA/Alg mixtures was defined by Oerther *et al.*[54], to obtain a gel mimicking the hydrated and viscoelastic environment afforded by the extracellular matrix embedding the chondrocytes. When this gel is prepared as a sponge, both a microporous network and an open macroporous one are obtained. Miralles *et al.*[40] have shown that the existence of such a macroporous network is in favor of cell colonization and is well adapted to the *in vitro* biosynthesis of an extracellular matrix.

In this study the macroscopic and histological observations were compared to specific biochemical parameters of cartilage, in order to appreciate the relevance of these latter as discriminating criteria of assessment.

The behavioural approach evidenced a normal progression of the animal weight, whatever the treatment, and a good tolerance to biomaterials which obviously did not induce any major handicap or/and inflammatory phenomena that can be visualized by biotelemetry.

However, in some groups, the synovial HA proved to significantly increase. According to the literature, excess of HA in the synovial fluid would be due an enhanced production by synoviocytes, in relation with articular inflammation[63,64]. Our results were consistent with this observation.

In fact, synovial HA is increased in the SR group, in concordance with the inflammatory process, widely reported to occur during the spontaneous repair[20,65,66]. It starts on the first week of the repair[66] and could be responsible for the weak stability of the neosynthesized tissue[2,63].

Significantly higher HA levels in Asp samples and, to a lesser extent, in AHAsp and HYBsp, can be correlated to recent works reporting on the stimulating effect of alginate on the production of IL-1 by monocytes[67]. In fact, even though alginate is considered as a fully compatible polymer[41], its mannuronic acid residues could stimulate the production of inflammatory cytokines (among which IL-1)[68]. This cytokine may activate the production of HA in the joint upon stimulation of endogenous cyclo-oxygenases[69].

In contrast, hydrogels only based on HA do not seem to cause this type of reaction. In fact, in certain conditions, HA has inhibitor effects on the production of IL-1 (air pouch model)[70] and may play a retro-control role on the production of this cytokine, in synergy with exogenous HA. Solchaga *et al.*[71] and Barbucci *et al.*[72] showed that, in addition, HA most often improves the biocompatibility of materials to which it is associated. These two elements,

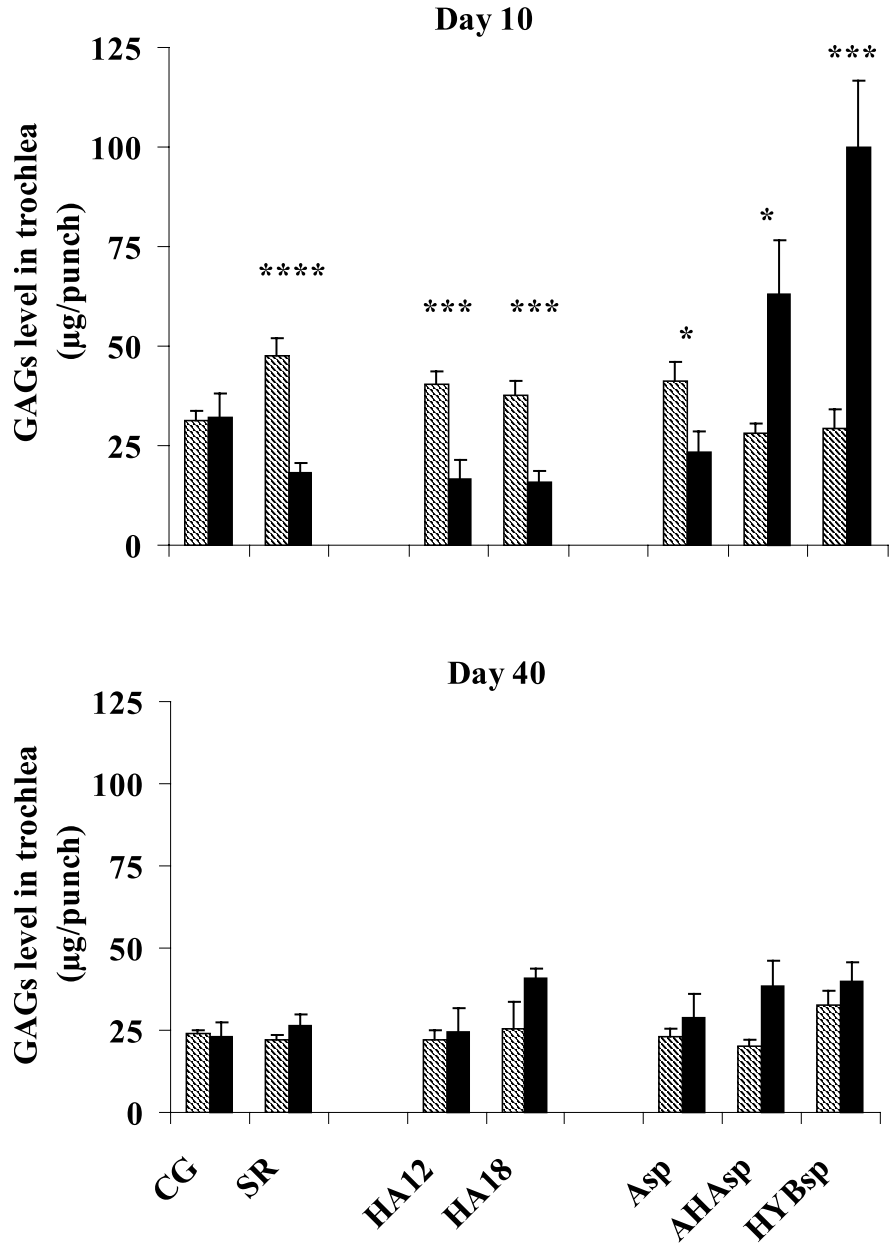

Fig. 5. GAGs measurements at D10 and D40 in trochlea of left and right knees for both groups. Analysis of GAGs is described in Materials and methods. Data (µg/punch) are expressed as a mean value±S.E.M. for $N=4$ from left (▨) and right knees (■) of both groups. Statistic analysis is performed by one-way ANOVA, to test the significant differences between right and left knees of each group with $P<0.05$*; $P<0.01$***; $P<0.001$****.

inhibition of IL-1 production and biocompatibility improvement, are in favor of better tissue reconstruction and scaffold integration.

The macroscopic aspect of the repair only accounts for the surface of a much deeper healing process. At day 10, during the inflammatory phase, a translucent, soft and gelatinous, fibrous tissue covers the surface of the defect. A similar aspect was previously reported by Kawamura[20]. This fibrous tissue starts forming from the defect's borders and progressively spreads inwards to fill the lesion, with a central zone looking like a crater. Once the surface was entirely covered, the soft tissue maturated and became white opaque[15,33]. As previously reported in another work, the repaired zone at the end of our study was usually clearly distinguishable from the native surrounding cartilage[15]. However, with certain samples, especially HA18, the repaired zone had a normal consistence and appearance.

As seen from the global histological results, the quality of the repair tissue and its integration to adjacent cartilage was sometimes disappointing. This fact is commonly reported in the literature, for studies with various materials or methods. For example, in agreement with the observation of Rahfoth *et al.*[35], we noted that the junction may vary from an intimate connection to a gap. Indeed, these critical factors could compromise the long-term evolution of the repair[66]. Since D10, numerous inflammatory or fibroblast-like cells colonized the gels, as confirmed by

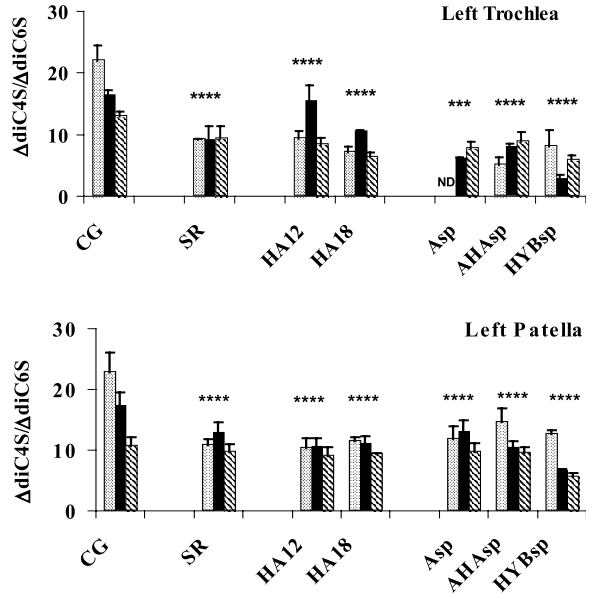

Fig. 6. Evaluation of ΔdiC4S/ΔdiC6S ratios in trochlea and patella facing it, of left knees for both groups, at D10 (▨), D20 (■) and D40 (▧). Data are expressed as mean value±S.E.M. for N=4. Statistic analysis is performed by two-way ANOVA to test the significant difference between implanted or SR groups and control group (CG) with P<0.01*** P<0.001**** (ND: non determined).

DNA analysis in the repair area. Some authors already observed such an invasion with other hydrogels[15,18]. The hydrogel disappeared slowly and was replaced by a fibrous tissue which progressively turns to fibrocartilage or even ultimately to a hyaline tissue[20]. This sequence was similar to the spontaneous healing of acute cartilage defects[3,4,63], but extended to the whole implanted gel. Hunziker[5,63] has previously noticed this phenomena with another gel and a fibrin clot.

In the case of unseeded materials, the assessment of DNA content in repaired zones is unfortunately not a suitable criteria to discriminate which cellular type, chondrocytes or other cells involved in the matrix biosynthesis, has invaded the lesion or the material. Synoviocytes would be good candidates for this cell rehabitation and have already been proposed by Hunziker[5,49,63]. Enhanced hyaluronate levels observed in repaired zones filled by unseeded materials are in strong support of this hypothesis. Such an overproduction of hyaluronate has been evidenced by Lammi *et al.*[73] during the repair of deep lesions. The weaker variation noted for the HYBsp group is likely due to the preliminar *in vitro* matrix synthesis by autologous chondrocytes, leaving only little opportunity to neighboring cells to invade the pre-formed implant.

The rat model of cartilage lesion used in this work enabled us to compare the histological results with the quantitative evaluation of GAGs. After the massive cellular colonization of unseeded materials, the presence of proteoglycans in repaired zones is observed, whatever the type of material and the nature of invading cells[41], which obviously recover their capacity to synthesize proteoglycans[74]. In the sponges, cells could profit of the large porosity of these materials to synthesize a GAGs-enriched extracellular matrix. Indeed, Chen *et al.*[75] and Solchaga *et al.*[71] have reported that a macroporous open network allows for an abundant synthesis of extracellular matrix. Freed *et al.*[21] made the same observation with polylactic or polyglycolic sponges, where human or bovine chondrocytes proved capable of synthesizing a proteoglycan-rich matrix.

The presence of a sufficient amount of GAGs constitutes a strong advantage with respect to the long-term stability of the repair, as observed by Frenkel *et al.*[16] with a porous collagen matrix.

In our work, the association of a porous alginate sponge with HA, a biological agent involved in proteoglycan synthesis[76,77] and with the seeding of autologous chondrocytes, leads to an optimal repair. In fact, the GAGs-rich extracellular matrix was preliminary obtained *in vitro* without cell multiplication in monolayer cultures, in contrast with the majority of authors[16,17,20,33,35]. These conditions have for advantage to avoid the dedifferenciation of chondrocytes and their synthesis activity for a type I collagen matrix.

However, histological assessments as well as GAGs measurements seem insufficient to specifically estimate the quality of the repair tissue and its similarity with native cartilage. Very few works attempt to correlate the synthesis of chondroitin sulfates, and especially their 4S to 6S ratios, with the quality of the neo-formed tissue. This ratio, which depends on many parameters such as the species, the localization in cartilage[78] and the age[79], varies with the cartilage maturation, accompanied by a chondroitin 4S decrease[79]. However, the position 4 or 6 of the sulfate group is actually concerned by two sulfotransferases, the mechanisms of action of which are not yet well known[79].

This parameter actually appears in our study as an important indicator. It showed that, despite the macroscopic and histological encouraging results observed with HA18 and HYBsp, the cartilaginous matrix obtained has not yet reached its optimal composition 40 days after the implantation. This is in agreement with the conclusions of various authors on other biomaterials[80].

In opposition with what is commonly suggested in many works, our *in vitro* preparation of a re-implantable artificial cartilage (HYBsp group) did not allow to reach a normal tissue more rapidly. 40 days after the implantation, the composition in chondroitin 4S and 6S is still at variance with that of the native matrix. Moreover, this type of implant leads to metabolic modifications of the whole surrounding cartilage. This phenomenon could be attributed to the conditions of the preliminary culture, which could lead to an implant able to provoke a transient inflammatory reaction. This latter would induce some increase of circulating pro-inflammatory mediators, some of which are already well known to disturb the cartilage metabolism and especially the synthesis of proteoglycans[81].

The other unseeded repair biosystems have a lesser influence and only modify the chondroitin sulfates 4S to 6S ratio of the patella localized in front of the repaired zone. This modification is probably due to the enhanced metabolic activity in the articular cavity during the repair process[82].

Conclusion

The present work enabled us to compare macroscopic, histological and biochemical parameters, in order to evaluate different types of materials with a single experimental model of cartilage defect. It evidenced that, even though the macroscopic and histological criteria received positive appreciations for certain biomaterials, notably a HA-based hydrogel and a hybrid HA-enriched alginate sponge, the biochemical parameters should be considered as well to

Table II
*Chondroitins 4S and 6S valued in trochlea and patella issued from treated and no treated animals at D10, D20 and D40 of repair (*N*=4 per group)*

	Days	Trochlea						Patella					
		Δdi6S μg/punch		Statistics	Δdi4S μg/punch		Statistics	Δdi6S μg/mg		Statistics	Δdi4S μg/mg		Statistics
		Right	Left	Right vs left	Right	Left	Right vs left	Right	Left	Right vs left	Right	Left	Right vs left
CG	10	1.53	1.49		32.44	31.17		2.23	2.34		45.53	49.15	
		0.03	0.38		2.76	6.04		0.52	0.52		9.18	4.93	
	20	1.18	1.07	NS	21.64	19.33	NS	2.52	2.80	NS	43.65	42.11	NS
		0.11	0.07		1.1	1.56		0.41	1.01		4.38	8.27	
	40	0.88	1.02		11.04	12.32		5.61	6.19		68.76	64.19	
		0.02	0.12		0.51	1.89		0.98	0.95		10.22	9.7	
SR	10	1.49	0.87		31.26	7.93		2.14	4.27		51.49	44.19	
		0.31	0.22		5.40	1.96		0.26	0.69		4.34	3.69	
	20	0.88	1.11	NS	14.22	10.23	**	2.61	3.18	***	43.92	38.33	NS
		0.17	0.07		2.51	3.37		0.45	0.52		10.89	2.99	
	40	1.26	2.08		15.29	17.70		2.70	3.47		38.21	30.57	
		0.20	0.68		2.38	5.35		0.09	0.64		1.91	1.35	
HA12	10	1.71	1.55		39.90	14.61		2.96	4.42		52.93	44.59	
		0.11	0.19		2.74	4.65		0.34	0.32		6.79	3.43	
	20	2.14	2.63	NS	42.10	38.65	***	2.61	3.47	***	40.61	34.67	NS
		0.09	0.24		1.56	3.81		0.28	0.59		5.64	3.41	
	40	1.01	1.77		12.32	13.15		2.70	3.81		29.76	31.81	
		0.20	0.68		3.45	2.61		0.31	0.68		3.42	2.06	
HA18	10	0.92	0.52		16.09	3.75		2.44	5.08		54.13	58.36	
		0.07	0.05		0.68	0.84		0.24	0.53		6.74	7.57	
	20	0.68	0.90	NS	11.17	9.59	*	3.16	4.15	NS	47.76	50.12	NS
		0.04	0.10		1.33	0.86		1.17	1.60		12.39	12.52	
	40	0.59	2.01		5.60	11.73		3.45	3.77		44.07	32.81	
		0.01	0.77		0.53	3.05		0.21	0.33		5.32	3.70	
Asp	10	0.86	ND		10.54	ND		2.47	3.58		57.05	40.17	
		0.07			2.49			0.16	0.54		7.27	0.59	
	20	1.10	1.12		10.92	6.70		2.95	5.03		52.69	60.12	
		0.16	0.05	NS	2.01	0.30	ND	0.49	0.79	***	3.07	1.47	NS
	40	1.09	1.27		9.72	11.24		3.09	4.53		45.25	39.43	
		0.15	0.16		0.38	1.98		0.39	0.96		6.15	3.34	
AHAsp	10	0.97	1.39		15.25	7.49		3.16	4.50		62.97	58.08	
		0.06	0.32		0.67	2.79		0.39	1.09		4.90	8.15	
	20	1.60	1.60	NS	20.20	12.46	*	3.37	5.46	**	60.80	52.56	NS
		0.37	0.13		2.64	1.18		0.63	1.25		8.65	7.19	
	40	1.10	1.46		12.01	13.02		1.59	2.71		22.82	24.83	
		0.17	0.27		2.54	4.12		0.28	0.38		1.71	1.53	
HYBsp	10	1.78	1.21		22.60	11.14		1.50	2.53		25.16	31.79	
		0.21	0.18		1.31	4.50		0.16	0.14		2.08	2.27	
	20	1.75	1.12	**	13.72	3.33	****	2.20	4.25	****	22.86	27.37	NS
		0.07	0.28		1.78	1.64		0.28	0.23		2.68	1.29	
	40	3.32	2.31		18.08	13.28		2.30	3.42		22.10	19.24	
		0.62	0.27		3.73	1.41		0.11	0.12		0.56	2.84	

Data were analysed in a two-way ANOVA and significance of differences (right vs left) is set at $P<0.05$.
ND: non-determined; NS: non-significantly different; $P<0.05$*; $P<0.02$**; $P<0.01$***; $P<0.001$****.

appreciate properly the intrinsic quality of the repair tissue. The follow-up of endogenous hyaluronate and chondroitin sulfates, should be completed in the future by the qualitative and quantitative analysis of collagens in the neosynthesized tissue. In addition, the knowledge of local or systemic contents in pro-inflammatory cytokines such as TNF-α, IL-1 or IL-6, would be of great importance to explain the metabolic changes noted in the cartilaginous tissue during the repair process.

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